DESCRIPTION

CELL-DEATH INDUCING FACTOR, CELL AND ANIMAL EXPRESSING THE SAME AND METHOD OF REENING FOR ANTI-CELL-DEATH THERAPEUTIC REMEDY

Technical Field

The present invention relates to screening for an anti-cell-death factor for use in the treatment of a disorder caused by excessive cell-death and to screening for a therapeutic drug for a disorder caused by aberrant suppression of human HtrA2 cell-death.

Background Art

Apoptosis is a physiologically programmed suicide of a cell and plays a critical role in the development processes and maintenance of the homeostasis of every animal (Jacobson et al., 1997). The aberrant suppression of apoptosis is a feature of cancers and autoimmune diseases, while the excessive apoptosis is considered to be involved in neurodegenerative disease (Thompson, 1995). Caspases are a family of intracellular cysteine proteases, which are main molecules executing apoptosis (Thornberry and Lazebnik, 1998). Effector caspases, for example, caspase-3 and -7 are activated by being cleaved by an initiator caspase like caspase-9. Once activated, effector caspases enzymatically degrade various structural proteins and regulatory proteins, resulting in the phenotype of apoptosis.

Nonetheless, the inhibitors of apoptosis protein (IAPs) were originally found in an insect virus, baculovirus, they are proteins evolutionarily well conserved from insects to human and playing a key role in the regulation of apoptosis. (Deveraux and Reed, 1999; Miller, 1999). A number of human IAP family proteins, for example, XIAP, c-IAP1 and c-IAP2 are known to be strong inhibitory factors which directly bind to caspase-3, -7 and -9, thereby inhibiting their activities. (Deveraux, et al., 1997, 1998; Roy et al., 1997). Among IAPs described above, XIAP is the strongest inhibitory factor against caspases and apoptosis induced by the caspases (Deveraux and Reed, 1999). The structure of XIAP is characterized by a triple repeat structure of baculoviral IAP repeat (BIR) domains at N-terminal region and RING finger domain at C-terminal region. Structural-functional analysis shows that the second BIR domain (BIR2) in XIAP is sufficient to inhibit caspase-3 and -7, and that the region covering the third BIR domain (BIR3) specifically inhibits caspase-9 (Deveraux, et al.,

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1999; Sun et al., 2000). To summarize, these data suggest that BIR domains in IAP are essential for inhibiting caspase activity.

Recently, a novel protein called Smac/DIABLO has been identified. It has been found that this protein has a function to facilitate the activation of caspases by eliminating the inhibitory activity of IAP to caspases (Du et al., 2000; Verhagen et al., 2000). Smac is synthesized as a precursor protein consisting of 239 amino acids. Fifty five amino acids on N-terminus, which serves as a signal for export to mitochondria, is cleaved off after the protein is exported to mitochondria. In response to various apoptotic stimuli, the mature Smac is released into cytoplasm, and binds to IAP therein, which is a cytoplasmic protein, to inhibit its activity. By researches afterward, Smac had been proved to be bound to BIR2 and BIR3 in XIAP at its N-terminus that starts with a sequence AVPI. Further, an amino acid mutation made on N-terminus of Smac eliminates the inhibitory activity of Smac to XIAP. It has been found that the N-termini of some cell-death proteins, Reaper, Hid and Grim, in Drosophila are similar to those of Smac and inhibit the activity of IAP at their N-termini (Abrams, 1999; Srinivasula et al., 2001). Smac was believed to be a functional homolog of such a protein of Drosophila, however it had not been unclear whether Smac was the only one mammalian protein to bind to IAPs and inhibit their activities (Du et al., 2000; Holcik and Korneluk, 2001).

Mitochondria have an extremely important function in physiological cell-death (Green and Reed, (1998) Science 281, 1309-12). Various apoptotic stimuli increase the permeability of mitochondrial outer membrane, leading the release of various cell-death inducing proteins present in the intermembrane space. Such cell-death proteins include cytochrome c, caspase-2, -3, -7 and -9, apoptosis induction factors (AIFs) and the like (Kroemer and Reed, (2000) Nat. Med. 6, 513-9). Smac/DIABLO is a recently identified cell-death inducing protein derived from mitochondria, which is released from mitochondria into cytoplasm, directly binds to XIAP and inhibits its caspase inhibitory activity (Du et al., (2000) Cell 102, 33-42; Verhagen et al., (2000) Cell 102, 43-53).

In this way, caspases have conventionally been emphasized as a factor executing apoptosis. As the speculation that inhibition of caspases may lead to the suppression of apoptosis, therapeutic drugs for disorders associated with apoptosis has been developing by targeting inhibition of caspases. However, it is known that in some cases cell-death can not be suppressed by the inhibition of caspases, which depends on the apoptotic stimuli. Further, it has been found that in many cases of neurodegenerative diseases which are believed to be attributable to excessive apoptosis, cell-death occurs without using caspases. There was a possibility of existence of a novel protein associated with such active cell-death which needs

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no caspases.

Conventionally, HtrA serine protease family has been known to be one of serine protease families (Faccio et al., (2000) J. Biol. Chem. 275, 2581-8; Gray et al., (2000) Eur. J. Biochem. 267, 5699-710; Hu et al., (1998) J. Biol. Chem. 273, 34406-12; Savopoulos et al., (2000) Protein Expr. Purif. 19, 227-34). Among them, bacterial HtrA gene products have been best-characterized of all HtrA serine protease families, wherein Htr means "High temperature requirement". Htr endopeptidase is localized in bacterial periplasm, and its existence is indispensable for bacterial thermotolerance (Lipinska et al., (1988) Nucleic Acids Res. 16, 10053-67, (1990) J. Bacteriol. 172, 1791-7; Seol et al., (1991) Biochem. Biophys. Res. Commun. 176, 730-6). Moreover, it has recently been found that bacterial HtrA has a double function, serving as a chaperone at a normal temperature and as a proteinase at a high temperature (Speiss et al., (1999) Cell 97, 339-47).

Disclosure of the Invention

The subject of the present invention is to provide a novel protein involved in cell-death, which has never been known, and a gene encoding the protein, and further the use of the protein or the gene to screen for an anti-cell-death factor for use in the treatment of a disorder caused by excessive cell-death or to screen for a remedy for a disorder caused by aberrant suppression of cell-death, and the screening method thereof.

Our strenuous studies resulted in finding a protein which inhibits IAP activity but is other than Smac. Namely, we have found a protein having a molecular weight of 36,000, which binds to XIAP, clarified the physicochemical properties of this protein by an amino acid sequence and mass analysis, and further analyzed the cell-death inducing effect of this protein. As a result, we isolated and identified a novel cell-death inducing factor HtrA2(or Omi), a serine protease. We isolated and identified HtrA2 as a novel XIAP-binding protein and proved that HtrA2 inhibited XIAP in the same manner as Smac did.

Our analyzing approach was carried out by both an amino acid sequence analysis using Edman degradation of the immunoprecipitated products of XIAP overexpressed in a cultured cell and a tandem mass spectrometry using electrospray ionization (Faccio et al., 2000; Gray et al., 2000).

HtrA2 is a protein belonging to a serine protease family which is well conserved from bacteria to human, and in particular, human HtrA2 serine protease shows extensive homology to bacterial HtrA and another known human HtrA, L56, at C-terminal region of the protein (Hu et al., (1998) J. Biol. Chem. 273, 34406-12). HtrA2 protein has a region assumed to be a transmembrance region, trypsin-like catalytic domain and PDZ domain on the amino acid

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regions of 105-121, 182-330 and 390-445, respectively (Faccio et al., (2000) J. Biol. Chem. 275, 2581-8). HtrA2 was reported to be localized in a endoplasmic reticulum or nucleus (Faccio et al., (2000) J. Biol. Chem. 275, 2581-8; Gray et al., (2000) Eur, J. Biochem. 267, 5699-710), however we have found that HtrA2 is a mitochondrial protein.

As mature HtrA2 is released from mitochondria in response to apoptotic stimuli, it is present in the intermembrane space like cytochrome c and Smac in a normal state. The fact that 4 amino acids, AVPS, in the N-terminus of mature HtrA2 is quite similar to 4 amino acids, AVPI, in the N-terminus of Smac provides an evidence that these two proteins inhibit IAP in a quite similar manner (Chai et al., (2000) Nature 406, 855-62). Northern blot shows that the amount of expression of Smac messenger RNA is very low in the brain, placenta, lung, leukocyte and the like, in contrast, HtrA2 seems to be expressed relatively anywhere (Gray et al., (2000) Eur. J. Biochem. 267, 5699-710). Those facts suggest that these two types of proteins act complementary to each other in various tissues.

The precursor of HtrA2 is a 50 kDa protein, which is exported to mitochondria, and then is cleaved of its N-terminal amino acids to give a 36 kDa mature protein. The N-terminus of the precursor of HtrA2 is similar to that of Smac. The precursor of HtrA2 does not bind to IAP, but mature HtrA2 does in a similar way as Smac does. Mature HtrA2 is usually sequestered in mitochondria, but is released into cytoplasm upon apoptotic stimuli (cell-death stimuli). HtrA2 released into cytoplasm is to bind to XIAP and inhibit the caspase inhibitory activity of XIAP, helping apoptosis to occur. Further, overexpression of HtrA2 outside mitochondria in a cultured cell makes the cell round up to shrink and die, but neither increase of caspase activity nor suppression by caspase inhibitors is observed. However, this effect is eliminated by inhibiting serine protease activity. In such a way, a strange cell-death which is independent on caspase overexpressed and is dependent on serine protease is triggered. As stated above, we have found that HtrA2 facilitates or induces cell-death by two different mechanisms: the one accompanied by the significant increase of caspase activity provided by IAP-inhibiting effect through the direct binding to IAP and the other which is independent on both IAP inhibition and caspase but is dependent on serine protease. Recently, it is pointed out that a non-apoptotic and caspase-independent cell-death is likely involved in cell-death in cancers and neurodegenerative diseases, and draws much attention (Kitanaka and Kuchino, (1999) Cell Death Differ. 6, 508-15; Sperandio et al., (2000) Proc, Natl. Acad. Sci. USA 97, 14376-81).

These data show that HtrA2 is, on one hand, an IAP inhibitory factor that is similar to Smac which has an effect of inducing serine protease-dependent cell-death, but on the other hand, a new type cell-death inducing factor which causes cell-death in two modes: using

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pathways with or without caspases.

Further, we have found a screening for an anti-cell-death factor for use in the treatment of a disorder caused by excessive cell-death using this novel cell-death inducing factor, HtrA2, in other words, an anti-cell-death therapeutic agent, its screening method, and a method for screening for a remedy for a disorder caused by aberrant suppression of cell-death, and completed the present invention.

Namely, the present invention is as follows.

(1)   A human HtrA2 protein to screen for an anti-cell-death factor for use in the treatment of a disorder caused by excessive cell-death.

(2)   The human HtrA2 protein according to the aspect (1), wherein the disorder caused by excessive cell-death is selected from a group consisting of neurodegenerative disease, cerebral ischemia, myocardial infarction and AIDS.

(3)   The human HtrA2 protein according to the aspect (1) or (2), wherein the human HtrA2 protein comprises an amino acid sequence from the 134th to the 458th of the sequence as set forth in SEQ ID NO: 2.

(4)   A recombinant cell expressing a human HtrA2 protein to screen for an anti-cell-death factor for use in the treatment of a disorder caused by excessive cell-death, wherein the cell comprises human HtrA2 gene or a variant thereof.

(5)   The recombinant cell expressing a human HtrA2 according to the aspect (4), wherein the disorder caused by excessive cell-death is selected from a group consisting of neurodegenerative disease, cerebral ischemia, myocardial infarction and AIDS.

(6)   The cell expressing a human HtrA2 according to the aspect (4) or (5), wherein the human HtrA2 protein comprises an amino acid sequence from the 134th to the 548th of the sequence as set forth in SEQ ID NO: 2.

(7)   A method of screening for an anti-cell-death factor for use in the treatment of a disorder caused by excessive cell-death, wherein the method uses the human HtrA2 protein according to any one of the aspects (1) to (3).

(8)   A method of screening for an anti-cell-death factor for use in the treatment of a disorder caused by excessive cell-death, wherein the method uses the cell expressing a human HtrA2 protein according to any one of the aspects (4) to (6).

(9)   The method of screening for an anti-cell-death factor according to claim 7 or claim 8, wherein the disorder caused by excessive cell-death is selected from a group consisting of neurodegenerative disease, cerebral ischemia, myocardial infarction and AIDS.

(10)   The screening method according to any one of the aspects (7) to (9), wherein the screening is for a factor that inhibits serine protease activity of human HtrA2 protein.

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(11)　　A recombinant cell expressing a human HtrA2 protein to screen for a remedy for a disorder caused by aberrant suppression of cell-death, wherein the cell comprises human HtrA2 gene or a variant thereof.

(12)　　The recombinant cell expressing a human HtrA2 protein, which comprises human HtrA2 gene or a variant thereof, according to the aspect (11), wherein the disorder caused by aberrant suppression of cell-death is autoimmune disease or cancer.

(13)　　A recombinant animal expressing a human HtrA2 protein to screen for a therapeutic drug for a disorder caused by aberrant suppression of cell-death, wherein the animal comprises human HtrA2 gene or a variant thereof.

(14)　　The recombinant animal expressing a human HtrA2 protein, which comprises human HtrA2 gene or a variant thereof, according to the aspect (13), wherein the disorder caused by aberrant suppression of cell-death is autoimmune disease or cancer.

(15)　　A method of screening for a remedy for a disorder caused by aberrant suppression of cell-death, wherein the method comprises administering a test substance to the cell according to the aspect (11) or (12) and measuring release of an HtrA2 protein from mitochondria to cytoplasm.

(16)　　A method of screening for a remedy for a disorder caused by aberrant suppression of cell-death, wherein the method comprises administering a test substance to the animal according to the aspect (13) or (14) and measuring release of an HtrA2 protein from mitochondria to cytoplasm.

(17)　　The method of screening for a remedy for a disorder caused by aberrant suppression of cell-death according to the aspect (15) or (16), wherein the disorder caused by aberrant suppression of cell-death is autoimmune disease or cancer.

(18)　　The method of screening for a remedy for a disorder caused by aberrant suppression of cell-death according to any one of the aspects (15) to (17), wherein the HtrA2 released from mitochondria to cytoplasm is measured using an anti-HtrA2 antibody.

　　　　The present invention will be explained in detail below.

1.　Acquisition of HtrA2 Gene DNA

　　　　In the present invention, the DNA can be acquired following the method explained in literatures and widely known to those skilled in the art such as: J. Sambrook, E. F. Fritsch & T. Maniatis(1989): Molecular Cloning, a laboratory manual, second edition, Cold Spring Harbor Laboratory Press; and Ed Harlow and David Lanc (1988): Antibodies, a laboratory manual, Cold Spring Harbor Laboratory Press.

　　　　HtrA2 protein can be obtained by isolating and purifying a protein capable of binding to XIAP and collecting an approximately 36 kDa mature protein. This protein is sequenced

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by a widely known amino acid sequencing method of protein and an appropriate primer is designed from the obtained amino acid sequence. Then, the screening of cDNA library which has been made from RNA extracted from human cell or tissue can be carried out to obtain HtrA2 gene. Alternatively, HtrA2 can directly be amplified and obtained by RT-PCR.

As HtrA2 gene DNA, a DNA containing the nucleotide sequence as set forth in SEQ ID NO: 1 or a DNA encoding the amino acid sequence as set forth in SEQ ID NO: 2 may be mentioned.

HtrA2 consisting of 458 amino acids that has the amino acid sequence as set forth in SEQ ID NO: 2 is a precursor HtrA2, and its N-terminal 133 amino acids which serves as a signal for export to mitochondria is cleaved off after the protein is exported to mitochondria to form a mature protein with 325 amino acids. HtrA2 protein for use in the present invention includes both precursor HtrA2 protein and a mature protein having the amino acid sequence from the 134th amino acid to the 458th amino acid of SEQ ID NO: 2.

The variant of HtrA2 gene DNA can be prepared by inducing a mutation into the cDNA of a wild type HtrA2 using a well known method. Variants of HtrA2 gene DNA include the following: a DNA capable of hybridizing with a DNA containing the nucleotide sequence as set forth in SEQ ID NO: 1 under a stringent condition and encoding a protein having the activity of HtrA2 protein; a protein comprising an amino acid sequence that is the amino acid sequence as set forth in SEQ ID NO: 2 in which one or more amino acids are deleted, substituted or added as well as having the activity of HtrA2 protein after the amino acids from the 1st amino acid to the 133rd amino acid of SEQ ID NO: 2 being cleaved off; or a protein comprising an amino acid sequence that is the amino acid sequence as set forth in SEQ ID NO: 2 in which one or more amino acids in the sequence from the 134th amino acid to the 458th amino acid are deleted, substituted or added as well as having the activity of HtrA2 protein. Herein, "stringent condition" means a condition which provides a specific hybridization, but not a nonspecific hybridization. For example, a condition which allows hybridization between DNAs having a high homology to each other, in other words, between DNAs having a homology of 60% or higher, preferably 80% or higher, but not between nucleic acids having a lower homology than that may be mentioned. In more particularly, it's a condition wherein a sodium concentration is from 150 to 900 mM, preferably from 600 to 900 mM, and the temperature is from 60 to 68°C, preferably at 65°C.

As an amino acid sequence that is the amino acid sequence as set forth in SEQ ID NO: 2 or the amino acid sequence from the 134th amino acid to the 458th amino acid of SEQ ID NO: 2, in which one or more amino acids are deleted, substituted or added, the following may be mentioned: the one that is the amino acid sequence as set forth in SEQ ID NO: 2 or the

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amino acid sequence from the 134th amino acid to the 458th amino acid of SEQ ID NO: 2, in which 1 to 10, preferably 1 to 5 and more preferably 1 or 2 amino acids are deleted; the one that is the amino acid sequence as set forth in SEQ ID NO: 2 or the amino acid sequence from the 134th amino acid to the 458th amino acid of SEQ ID NO: 2, in which 1 to 10, preferably 1 to 5 and more preferably 1 or 2 amino acids are substituted; or the one that is the amino acid sequence as set forth in SEQ ID NO: 2 or the amino acid sequence from the 134th amino acid to the 458th amino acid of SEQ ID NO: 2, in which 1 to 10, preferably 1 to 5 and more preferably 1 or 2 amino acids are added.

As such an amino acid sequence that is the amino acid sequence as set forth in SEQ ID NO: 2 or the amino acid sequence from the 134th amino acid to the 458th amino acid of SEQ ID NO: 2, in which one or more amino acids are deleted, substituted or added, those having a homology of at least 60% or higher, preferably 80% or higher and more preferably 95% or higher to the amino acid sequence as set forth in SEQ ID NO: 2 or the amino acid sequence from the 134th amino acid to the 458th amino acid of SEQ ID NO: 2 respectively may be mentioned. The homology can be calculated by using BLAST wherein, for example, the default, that is to say, initial setting parameters can be used.

Once the nucleotide sequence of the gene is determined, HtrA2 gene DNA or a variant thereof can be obtained by chemical synthesis afterward, by PCR using a cloned cDNA as a template or by hybridization using a DNA fragment having the nucleotide sequence as a probe. In this way, from the thus obtained DNA, a protein comprising an amino acid sequence that is the amino acid sequence as set forth in SEQ ID NO: 2 or the amino acid sequence from the 134th amino acid to the 458th amino acid of SEQ ID NO: 2, in which one or more amino acids are deleted, substituted or added, as well as having the activity of HtrA2 protein can be acquired.

Herein, "activity of HtrA2 protein" means a serine protease activity, and whether that activity is held or not can be determined by, for example, an in vitro protease assay using β casein as a substrate.

2. Acquisition of HtrA2 Protein and A Recombinant Cell Expressing HtrA2 Protein

A recombinant cell expressing HtrA2 Protein can be obtained by inserting the HtrA2 gene DNA of the present invention and a variant thereof into an expression vector and introducing the vector into an appropriate host cell. The DNA to use therein may be any of the followings: the full-length gene of HtrA2 as set forth in SEQ ID NO: 1, DNA encoding the amino acid sequence of SEQ ID NO: 2, DNA without the part encoding the amino acid sequence from the 1st amino acid to the 133rd amino acid of SEQ ID NO: 2, or DNA encoding the amino acid sequence from the 134th amino acid to the 458th amino acid of SEQ ID NO: 2.

The former two DNAs express precursor HtrA2 and the latter two DNAs express mature HtrA2. Alternatively, GST-coding DNA or hexahistidine-coding DNA may be ligated arbitrarily. As a vector, any vector such as plasmids, phages and viruses can be used as long as it can be replicated in a host cell. For example, E. coli plasmids such as pBR322, pBR325, pUC118, pUC119, pKC30 and pCFM536; Bacillus subtilis plasmids such as pUB110; yeast plasmids such as pG-1, YEp13 and YCp50; phage DNAs such as λgt110 and λZAPII may be used. As a vector for a mammalian cell, virus DNAs such as baculovirus, vaccinia virus and adenovirus; SV40 and derivatives thereof may be used. A vector comprises the origin of replication, selection marker and promoter, and may further comprise an enhancer, transcription-terminating sequence (terminator), ribosome binding site, polyadenylation signal and so on according to need. As a promoter, any promoter may be used as long as it can be expressed efficiently in a host cell. For example, promoters of SRα, SV40, LTR, CMV and HSV-TK may be used.

As a host cell, bacterial cells such as E. coli, Streptomyces and Bacillus subtilis; fungal cells such as Aspergillus strain; yeast cells such as bread yeast and methanol-utilizing yeast; insect cells such as Drosophila S2 and Spodoptera Sf9; mammalian cells such as HEK293T, HeLa cell, SH-SY5Y, CHO, COS, BHK, 3T3 and C127 may be used .

Transformation can be conducted by a well known method such as transfection mediated by calcium chloride, calcium phosphate and DEAE-dextran, and electroporation.

HtrA2 protein can be isolated from thus obtained cells expressingHtrA2 protein. For example, mature HtrA2 protein among isolated proteins and a cell expressing the mature protein, in particular animal cell, can be used for screening for an anti-cell-death factor for use in the treatment of a disorder caused by excessive cell-death, and further a cell expressing the precursor protein can be used for screening for a remedy for a disorder caused by aberrant suppression of cell-death.

3. Acquisition of Anti-HtrA2 Protein Antibody

Using HtrA2 protein as an antigen, ananti-HtrA2 protein antibody can be produced following a method well known to those skilled in the art, for example, by inoculating the protein into animals such as mouse, guinea pig, rabbit or goat subcutaneously, intramuscularly, intraperitonealy or intravenously for several times for sufficient immunization followed by collecting the blood from the animals to separate the serum. At that time, appropriate adjuvants can also be used. The monoclonal antibodies may also be produced by a well known method. For example, a hybridoma is prepared by fusing a splenocyte of HtrA2 protein-immunized mouse with a mouse myeloma cell, and then a monoclonal antibody can be prepared from the culture supernatant of the hybridoma or the ascites of mice

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intraperitoneally administered the hybridoma. HtrA2 protein for use as an immunizing antigen may be a natural protein or a recombinant protein, and also be a chemically synthesized one. Further, a protein having a whole amino acid sequence as well as a peptide fragment having a part of structure of the protein and a fusion protein with other protein may be used. Referring to a peptide fragment, a fragment prepared by degrading the protein with an appropriate proteinase, and an expression product of an expression vector into which the nucleotide sequence as set forth in SEQ ID NO: 1 has been integrated in whole or part may be used. Meanwhile, when mature HtrA2 protein released from mitochondria to cytoplasm is to be measured, it is desired to use mature HtrA2 protein or the fragment thereof as an immunizing antigen. A polypeptide fragment can also be used after chemically binding to an appropriate carrier protein. The reactivity of the obtained antibody can be determined by a method well known to those skilled in the art, such as enzyme immunoassay (EIA), radioimmunoassay (RIA) and western blotting.

4. Acquisition of An Animal HtrA2-Expressing Model

To a gene to be introduced into an HtrA2-expressing model animal, a promoter sequence or an enhancer sequence should be ligated to control its expression. Referring to these sequences, there is no limitation in particular, and usual sequences can be used in combination arbitrarily. In such a case, a DNA to be introduced may be any one of the followings: the full-length gene of HtrA2 as set forth in SEQ ID NO: 1, DNA encoding the amino acid sequence of SEQ ID NO: 2, DNA without the part encoding the amino acid sequence from the 1st amino acid to the 133rd amino acid of SEQ ID NO: 2, or DNA encoding the amino acid sequence from the 134th amino acid to the 458th amino acid of SEQ ID NO: 2. When mature HtrA2 protein released from mitochondria to cytoplasm is to be measured, the former two DNAs are preferred to use.

Transgenic animals can be prepared following a method described in, for example, Pro. Natl. Acad. Sci. USA 77: 7380-7384, 1980, wherein the transgene described above is introduced into a mammalian totipotent cell, the cell is developed into an individual, and then an individual in which the transgene has been integrated into the genome in its somatic cell is selected. For a mammal, mice are preferably used because many inbred lines have been created and techniques such as cultivation of oocytes and external fertilization have been established for them, but it is technologically possible to use all kinds of animals as a subject. As a totipotent cell into which the gene is introduced, in the case of a mouse, a cultured cell such as a multipotent ES cell can be used as well as an oocyte or an early embryo. As a method for introducing a gene into a cultured cell, widely known methods using electrostatic pulse, liposome, calcium phosphate and the like can be used, and when considering the

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production efficiency of an individual transgenic animal and the transferring efficiency of a transgene to the next generation, physical injection of a DNA solution into an oocyte (microinjection) is preferred.

An HtrA2-expressing animal can be obtained by implanting a totipotent cell injected with a gene into the oviduct of a tentative mother and developing it into an individual. The presence of the transgene can be confirmed using DNA extracted from somatic cells by Southern blot analysis, PCR assay and the like. An animal in which HtrA2 gene or a variant thereof is steady integrated into the part of its chromosome can be created efficiently by using the individual in which the presence of the transgene is confirmed as a founder and mating them.

5. Screening for Anti-Cell-Death Therapeutic Agent

The mature HtrA2 protein and mature HtrA2 protein-expressing cell described above can be used to screen for an anti-cell-death factor for use in the treatment of a disorder caused by excessive cell-death. As a disorder caused by excessive cell-death, neurodegenerative disease, cerebral ischemia, myocardial infarction, AIDS and the like may be mentioned.

An anti-cell-death factor for use in the treatment of a disorder caused by excessive cell-death can be selected by mixing mature HtrA2 protein with a solution extracted from various tissues of an organism and screening for a protein capable of inhibiting the serine protease activity of HtrA2 protein. Further, an anti-cell-death factor for use in the treatment of a disorder caused by excessive cell-death can be isolated by selecting a protein capable of binding to HtrA2 protein and analyzing the function of the protein. Alternatively, a cell expressing, preferably overexpressing, mature HtrA2 protein may also be used.

6. Screening for A Remedy for A Disorder Caused by Aberrant suppression of Cell-Death

An HtrA2 protein-expressing cell or transformed animal can be used to screen for a remedy for a disorder caused by aberrant suppression of cell-death. As a disorder caused by aberrant suppression of cell-death, autoimmune disease, cancer and the like may be mentioned. In such a case, it is desired to integrate a gene encoding precursor HtrA2 into a cell to be used, and HtrA2 protein should be expressed so as to be localized in mitochondria. Then, it is possible to screen for a remedy for a disorder caused by aberrant suppression of cell-death by administering a test substance to the cell or animal and using the activity of releasing HtrA2 protein from mitochondria as an indicator. The activity of releasing HtrA2 protein from mitochondria can be determined, for example, by measuring HtrA2 in cytoplasma which was released from mitochondria using the anti-HtrA2 antibody.

Brief Description of the Drawing

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Figure 1 shows the results of SDS-PAGE of HtrA2.

Figure 2 shows the reverse phase chromatogram of HtrA2.

Figure 3 shows the full-length amino acid sequence of human HtrA2.

Figure 4 shows the binding of endogenous XIAP to endogenous HtrA2.

Figure 5 shows the binding of mature HtrA2 to a IAP family protein.

Figure 6 is a schematic diagram showing full-length HtrA2 and mature one.

Figure 7 shows the binding of recHtrA2 (AVPS) and recHtrA2 (MVPS) to BIR2 and BIR3 domains in XIAP.

Figure 8 shows the processing of caspase 3 and acting sites of BIR2 domain in XIAP (XIAP-BIR2) and BIR3+RING domains in XIAP (XIAP-BIR3+RING).

Figure 9 shows the effects of recHtrA2 (AVPS) and recHtrA2 (MVPS) on both inhibitory effects of BIR2 in XIAP (GST-BIR2) to caspase 3 activity and of BIR3 in XIAP (GST-BIR3) to caspase 9 activity.

Figure 10 shows whether inhibitory effects of XIAP to the serine protease activity of HtrA2 are appeared or not.

Figure 11 shows the cell fractionation of HeLa cell.

Figure 12 is a photograph showing the translocation of HtrA2 and cytochrome c (Cyt c) to a cytoplasmic fraction after UV irradiation.

Figure 13 shows the results of immunostaining of HtrA2 and cytochrome c (Cyt c).

Figure 14 shows the results of Western blot analysis of HtrA2 showing that in the spinal cord of a familial ALS model mouse, HtrA2 is released into cytoplasm in accordance with aging (1-, 3- and 5-month after birth).

Figure 15 shows that HtrA2 increased caspase activity (DEVDase activity) caused by UV irradiation.

Figure 16 is a photograph showing an untypical cell-death induced by mature HtrA2.

Figure 17 shows that protease activity of mature HtrA2 is required for induction of an untypical cell-death.

Figure 18 shows whether the significant increase of caspase activity (DEVDase activity) by mature HtrA2 is appeared or not.

Figure 19 shows whether a caspase inhibitor affects the HtrA2 inhibition effect or not.

Sequence Listing

SEQ ID NOs: 3 and 4: Synthesis

Description of the Preferred Embodiment

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Next, the present invention may specifically be explained by way of Examples, but these examples are not intended to limit the present invention.

In Examples of the present invention, transfection, co-immunoprecipitation and immunoblot analysis were carried out by the methods described below.

[Transfection, Co-immunoprecipitation and Immunoblot Analysis]

HEK293 and HeLa cells were transfected by LipofectAMINE reagent (GIBCO-BRL). For co-immunoprecipitation, 2×10^6 HEK293 cells were plated in a 10 cm dish, and on the next day, they were transfected with 5 µg of FLAG-tagged or Myc-tagged IAP expression vector and 5 µg of HA-tagged HtrA2 expression vector. At 24 hours after transfection, the culture medium was changed to the one containing 5 µM of proteasomal inhibitor (MG132, Peptide Institute), and after culturing for further 16-hour, the cell extract was prepared. IAPs in the cell extract were immunoprecipitated with an anti-FLAG M2 antibody (Sigma) or an anti-Myc 9E10 antibody (Santa Cruz biotechnology), subjected to SDS-PAGE, and transferred onto PVDF membrane (Immobilon supplied by Millipore). Subsequently, the co-immunoprecipitates were subjected to immunoblotting analysis with anti-HA3F10 antibody (Roche Diagnostics). For detecting the primary antibody, ECL kit (Amersham Pharmacia) was used.

Further, a recombinant protein was prepared by the method described below.

[Recombinant Protein]

GST-tagged XIAP protein was prepared by the method described in Suzuki et al., (2001) J. Biol. Chem. 276, 27058-63. C-terminal hexahistidine-tagged HtrA2 and its variants were subcloned into pET28a plasmids, and recombinant proteins were prepared by the method described in Savopoulos et al., 2000 Protein Expr. Purif. 19, 227-34. The concentration of the proteins was measured with Coomassie Plus Protein Assay Reagent Kit or Micro BCA Protein Assay Reagent Kit (Pierce).

Cell fractionation and immunostaining were conducted as follows.

[Cell Fractionation]

HeLa cell was suspended in a 3 volume of buffer A (20 mM Hepes-KOH[pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 250 mM sucrose) followed by passing through a 29-gauge injection needle ten times and subjected to 10-minute centrifugation at 750 x g twice to collect the supernatant. Then, the supernatant was centrifuged for 15 minutes at 10,000 x g to obtain a precipitate that was called heavy membrane fraction. The supernatant was further centrifuged for an hour at 100,000 x g to obtain a precipitate that was called light membrane fraction and a supernatant that was called

cytoplasmic fraction.

[Immunostaining]

HeLa cell was subcultured in 8-well chamber slides, and from 24 to 48 hours later, the cell was washed with PBS, immobilized with 4% paraformaldehyde, made into water-permeable with 0.2% Triton X-100, added with anti human HtrA2 antiserum (1:200 dilution) and cytochrome c antibody (PharMingen, 1:500 dilution), and incubated for an hour at room temperature. The primary antibody was detected by the secondary antibody tagged with Alexa 488 or 546 (Molecular Probes) and analyzed by confocal laser scanning microscope analysis system (Fluoview, supplied by Olympus).

Further, the determination of caspase activity and cell-death assay were conducted by the method described below.

[Determination of Caspase Activity and Cell-Death Assay]

2×10^6 HeLa cells or HEK293 cells were plated in 6-well dishes, and on the next day, transfected with from 1 to 3 μg of plasmid DNA together with 0.2 μg of pEGFP N3 (Clontech). After 24 hours, the HeLa cells were irradiated with ultraviolet ray to prepare the cell extract. The caspase activity in the cell extract was determined by the method described in Suzuki et al., (2001) Proc. Natl. Acad. Sci. USA 98, 8662-7. Cell-death assay was conducted by culturing the transfected HEK293 cell for from 24 to 36 hours and measuring the ratio of the cells with a round or condensed shape in GFP-positive cells.

[Example 1]

Identification and Cloning of HtrA2

(1) Purification of XIAP Binding Protein

293T cells transfected with pcDNA3-FLAG-XIAPΔRING were suspended in a lysis buffer (10 mM Tris [pH 8.0], 120 mM NaCl, 5 mM EDTA) and the soluble fraction was extracted. From this fraction, FLAG-XIAPΔRING protein was immunoprecipitated using anti-FLAG M2 antibody. After washing the precipitate with lysis buffer five times, FLAG-XIAPΔRING protein and its binding protein was eluted with 0.1 M glycine-HCl (pH 2.8). The effluent was separated by SDS-PAGE, and then the bands detected by CBB staining were excised and purified.

(2) HPLC-MS/MS and Amino Acid Sequencing

In-gel digestion was carried out by the method described in Kawasaki et al., (1999) Anal. Biochem, 191, 332-6. Concretely, the 36 kDa band detected by CBB staining was treated in 50 mM Tris-HCl (pH 9) containing 0.1 μg of Achromobacter Protease I (Lys-C) and 0.1% SDS for 12 hours at 37°C, then the amino acid sequence of a degraded product extracted from the gel was determined by HPLC-MS/MS and Edman degradation.

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(3) Preparation of Plasmid

The plasmids encoding FLAG-tagged wild type XIAP and RING finger-deleted XIAP (pcDNA3-FLAG-XIAP, pcDNA3-FLAG-XIAPΔRING) were prepared by the method described in Suzuki et al., (2001) Proc. Natl. Acad. Sci. USA 98, 8662-7. pcDNA3-FLAG-c-IAP1 and pcDNA3-FLAG-c-IAP2 were prepared by integrating respective EcoRI-XhoI fragments of RT-PCR products of pcDNA3-Myc-c-IAP1 and pcDNA3-Myc-c-IAP2 into the EcoRI-XhoI sites of pcDNA3-FLAG-N. Plasmids encoding Myc-tagged IAP containing pcDNA3-Myc-survivin, pcDNA3-Bax and pCMV-Fas plasmids were provided by Doctor John C. Reed (Burnham Institute). DNA encoding human HtrA2 was amplified by RT-PCR using the following primers. Forward primer: ccgcaattggccATGGCTGCGCCGAGGGCG (SEQ ID NO: 3) and reverse primer: ctctcgagTTCTGTGACCTCAGGGGTC (SEQ ID NO: 4). MfeI-XhoI fragments of the PCR products were inserted into the MfeI-XhoI site of pcDNA3-Myc-C and pcDNA3-HA-C plasmids respectively to prepare pcDNA3-HtrA2-Myc and pcDNA3-HtrA2-HA. Plasmids encoding N-terminal deleted HtrA2 used in Examples herein were prepared in the same manner. Further, point mutants were prepared using Quick Change site-directed mutagenesis kit (Stratagene). Plasmid encoding Myc-tagged human Smac/DIABLO was prepared by inserting the RT-PCR product into the BamHI-XhoI site of pcDNA3-Myc-C. It was confirmed by conducting DNA sequencing that all the plasmids was prepared accurately.

(4) Identification of HtrA2 and Acquisition Thereof

To identify a novel XIAP-binding protein, a cell extract was prepared from a 293T cell transiently transfected (genetically introduced) with a variant having FLAG tag on N-terminus and lacking RING finger motif (XIAPΔRING). Thereafter, the cell extract was mixed with agarose beads which had been conjugated with an anti-FLAG monoclonal antibody. XIAPΔRING was used because it expresses more efficiently than full-length XIAP. After washed thoroughly, the binding protein was eluted under an acidic condition and separated by SDS-PAGE gel. The bands of two kinds of protein, 23 kDa and 36 kDa, were observed on a Coomassie-stained gel (Fig. 1). In Fig.1, the arrowhead indicates the FLAG-XIAPΔRING and bands A and B show proteins eluted together with FLAG-XIAPΔRING. These bands were not observed with the control FLAG-tagged protein.

As a 23 kDa band was assumed to be Smac, a 36 kDa band was expected as an important novel protein, and In-gel digestion was conducted with Achromobacter Protease I (a.k.a. Lys-C). The excised peptide was collected from the gel, then separated by RP-HPLC and sequenced by electron ion spray tandem mass spectrometry. As uncertain MS/MS spectra could not be determined by database searching, the amino acid sequence analysis was

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conducted by Edman degradation. The amino acid sequence of fraction 1 shown in Fig. 2 was perfectly consistent with that of an N-terminal fragment of mature human HtrA2 (Faccio et al., (2000) J. Biol. Chem. 275, 2581-8; Gray et al., (2000) Eur. J. Biochem. 267, 5699-710). Moreover, based on that information, another analysis was conducted by tandem mass spectrometry resulting in finding that seven fragments obtained by cleaving with Lys-C covered all of the sequences of the mature type of an alternative splicing product called HtrA2 Type 13B (Figs. 2 and 3) (Gray et al., 2000 Eur. J. Biochem. 267, 5699-710). Fig. 2 shows the elution results of the reverse phase HPLC conducted on the protein that is shown as A in Fig. 1 which had been degraded by Achromobacter Protease I (Lys-C), showing that the amino acid sequence obtained from the Edman degradation of peak 1contained the amino terminal fragment of mature human HtrA2 (Fig. 3, underline). The peaks 2, 3, 4 and 5 in Fig. 2 were consistent with 325-347, 238-324, 349-395 and 157-237 of human full-length HtrA2 respectively. Fig. 3 shows the full-length amino acid sequence of human HtrA2 (Type 13B). In Fig. 3, the amino acid sequence of peak 1 shown in Fig. 2 was underlined. The arrowhead indicates a cleavage site to produce mature HtrA2. Although human HtrA2 Type 13B is a 458 amino acid protein, after exported to mitochondria, its N-terminal 133 amino acids are cleaved off, and as a result, HtrA2 Type 13B becomes 36 kDa. Therefore, our analyzed band is the same as mature HtrA2 also in size.

[Example 2]

Preparation of Anti-human HtrA2 Antibody

The antiserums to human HtrA2 and human XIAP were prepared by preparing both C-terminal hexahistidine-tagged recombinant HtrA2 protein and N-terminal GST-tagged recombinant XIAP-BIR1 protein by the method described in Suzuki et al., (2001) J. Biol. Chem, 276, 27058-63, and then immunizing rabbits with these protein antigens respectively.

[Example 3]

Binding to IAPs of HtrA2 precursor and mature HtrA2

As shown in Fig. 1, endogenous HtrA2 co-precipitated with overexpressed XIAPΔRING. Then, we detected the binding of HtrA2 binds to XIAP within a cell. HEK293 cell extract (1 mg protein) was incubated together with control antibody or anti-XIAP antibody which was conjugated to protein G for 4 hours at 4°C. Then their immunoprecipitates were separated by SDS-PAGE, transferred onto PVDF membrane, and then analyzed by immunoblotting with anti-HtrA2 antibody or anti-XIAP antibody. As shown in Fig. 4, endogenous HtrA2 co-precipitated with endogenous XIAP as well, strongly supporting the idea that the binding of XIAP to HtrA2 has a significant meaning also in physiological state. In Fig. 4, the arrowheads indicate heavy and light chains of IgG.

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Next, to examine the binding of an IAP family protein to HtrA2, various proteins that are IAP, XIAP, c-IAP1, c-IAP2, surviving and the like, which were tagged with FLAG or Myc at their N-terminus, were used for transient transfection together with plasmid encoding C-terminal HA-tagged human HtrA2 protein. IAP tagged with FLAG at its amino terminus, and IAP tagged with Myc at its amino terminus were coexpressed in HEK293 cell together with HtrA2 tagged with HA at its carboxyl terminus. Then, the whole cell extract and the immunoprecipitate obtained by using an anti-FLAG antibody or an anti-Myc antibody (respectively, IP: FLAG and IP: Myc) were analyzed by immunoblotting using an anti-FLAG antibody, an anti-Myc antibody or an anti-HA antibody. As a result, while 50 kDa HtrA2 precursor protein did not co-precipitate with any IAP, 36 kDa mature HtrA2 protein co-precipitated with XIAP, c-IAP1 and c-IAP2. Mature HtrA2 did not bind to survivin, while it was reported that Smac bound to it (Du et al., (2000) Cell 102, 33-42) (Fig. 5). These results indicate that mature HtrA2 tends to bind to IAP proteins which have strong caspase inhibitory activity, such as XIAP, c-IAP1 and c-IAP2 (Deveraux, et al., (1997) Nature 388, 300-4; Roy et al., (1997) EMBO J.16, 6914-25; Tamm et al., (1998) Cancer Res. 58, 5315-20).

[Example 4]

Inhibition of Caspase Inhibitory Activity of XIAP by HtrA2

Production of mature HtrA2 requires a cleavage of a sequence preceding the alanine at position 134 (Savopoulos et al., (2000) Protein Expr. Purif. 19, 227-34) (Fig. 6). In Fig. 6, transmembrance region (TM), trypsin-like active site and PDZ domain were presented with respectively corresponding amino acid numbers. S306 means a serine residue in the active site. RecHtrA2 (AVPS) and recHtrA2 (MVPS) respectively represent wild type and amino terminus-mutated recombinant type of mature HtrA2 protein. The amino terminal sequences were presented in parentheses. The sequence of the first four amino acids of mature HtrA2 is AVPS. This sequence is quite similar to N-terminal sequence of Smac that is AVPI. Smac binds to BIR2 and BIR3 domains of XIAP, so its N-terminal sequence should not be mutated nor modified and be maintained to be normal (Chai et al., (2000) Nature 406, 855-62). Moreover, it has been exhibited that molecules having an N-terminal sequence similar to that of Smac, like a small subunit of caspase-9, bind to XIAP (Srinivasula et al., (2001) Nature 410, 112-6). These results suggest that mature HtrA2 binds to XIAP in the similar manner to Smac did and inhibit its activity (Chai et al., (2000) Nature 406, 855-62). To examine this suggestion, we prepared E. coli recombinant proteins of both wild type mature HtrA2 and mutant type one in which the most N-terminal alanine was mutated to methionine. These proteins were respectively named recHtrA2 (AVPS) and recHtrA2 (MVPS) and served to binding experiments to fusion proteins of wild type XIAP and various variant XIAPs with

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glutathione S-transferase (GST). After the pull-down reaction of both wild type and amino terminal-mutated type of recombinant HtrA2 protein with GST-tagged recombinant XIAP protein, they were analyzed by immunoblotting with an anti-pentahistidine antibody and an anti-GST antibody. As expected, while recHtrA2 (AVPS) binds to BIR2 and BIR3 domains of XIAP, recHtrA2 (MVPS) did not bind to XIAP at all (Fig. 7). In Fig. 7, the arrowhead indicates hexahistidine-tagged recombinant HtrA2 protein. The way of binding of HtrA2 to XIAP is similar to that of Smac to XIAP. Therefore, to determine whether HtrA2 which is similar to Smac attenuates the inhibitory effects of XIAP to caspase, we examined whether recHtrA2 (AVPS) and recHtrA2 (MVPS) increase the activity of caspase-3 or facilitate the activation of caspase-3. As an experimental system, we used a cell-free system which allows caspase-3 to activate in vitro by adding cytochrome c to 293 cell extract (Deveraux et al., (1997) Nature 388, 300-4). BIR3+RING domain directly inhibits active caspase-9 (Deveraux et al., (1999) EMBO J. 18, 5242-51), and thereby prevents procaspase-3 from being cleaved into large subunit and small subunit and activated in vitro. On the contrary, BIR2 domain directly inhibits caspase-3, and thereby prohibits caspase-3 from producing p19 and p17 from large subunit p20 by autodegradation (Deveraux et al., (1997) Nature 388, 300-4) (compare Fig. 8 to Lane 4, 7 and 10 in Fig. 9). Fig. 8 shows the processing of caspase 3 and acting sites of BIR2 domain in XIAP (XIAP-BIR2) and BIR3+RING domains in XIAP (XIAP-BIR3+RING). Caspase 3 (P32) is processed into P20 and P12 by caspase 9. XIAP-BIR3+RING inhibits this process. Further, p20 of caspase 3 is processed into p19 and p17 by caspase 3 itself. XIAP-BIR2 inhibits this process. Caspase 3 was activated by adding cytochrome c and dATP to the cytoplasmic fraction of HEK293 cell in the presence or absence of each purified protein. The activation of caspase 3 was detected by immunoblotting analysis using an anti-caspase 3 antibody. RecHtrA2 (AVPS) suppresses the processing of caspase-3 induced by BIR2 and BIR3+RING, while recHtrA2 (MVPS) did not exhibit such effects (Fig. 9). This indicates that recHtrA2 (AVPS) inhibits both the inhibitory effect of BIR2 in XIAP (GST-BIR2) to caspase 3 activity and the inhibitory effect of BIR3 in XIAP (GST-BIR3) to caspase 9 activity, but recHtrA2 (MVPS) does not. In Fig. 9, a white arrowhead indicates a band, which crossreacts with an anti-caspase 3 antibody, resulting from a trace amount of contamination from E. coli. These results indicate that HtrA2 inhibits the caspase inhibitory activity of XIAP in a quite similar manner to Smac.

[Example 5]

Studies on the Inhibition of Serine Protease Activity of HtrA2 by XIAP

To examine whether XIAP inhibits the serine protease activity of HtrA2 or not, in vitro protease assay was conducted using β-casein as a substrate. β-casein (6 μM) was

incubated with 5 nM of HtrA2 recombinant protein in the presence or absence of GST-XIAP recombinant protein, then 1 or 2 hours later, the samples were separated by SDS-PAGE and CBB-stained. The recombinant mature HtrA2 completely cleaves 50 nM of β-casein at 37°C for 2 hours (Fig. 10). XIAP did not inhibit HtrA2 from cleaving β-casein in vitro even at a concentration as high as 2 µM. The results indicate that XIAP binds to mature HtrA2 but does not exhibit inhibitory activity to it.

[Example 6]

Release of HtrA2

HtrA2 was postulated to be a mitochondrial protein by PSORT analysis, however some previous reports noted that HtrA2 existed in endoplasmic reticulum or nucleus (Faccio et al., (2000) J. Biol. Chem. 275, 2581-8; Gray et al., (2000) Eur. J. Biochem. 267, 5699-710). In addition to it, it had been found that HtrA2 had an effect similar to that of Smac which is a mitochondrial protein, so the possibility of intracellular localization of HtrA2 was reexamined. In the cell fractionation of HeLa cell, HtrA2 was collected into the heavy membrane fraction like cytochrome c, a mitochondrial protein (Fig. 11). HtrA2 was detected together with cytochrome c (Cyt c) in a heavy membrane fraction (HM) which contained mitochondria. Protein disulphide isomerase (PDI) and XIAP were used as indicators for light membrane fraction (LM) and cytoplasmic fraction (CE) respectively (Fig. 11). Immunostaining of HeLa cell with the antiserum which was prepared against recombinant HtrA2 indicated that HtrA2 and cytochrome c co-localized in mitochondria. However, when inducing apoptosis by UV stimulation, both two proteins, HtrA2 and cytochrome c, shows a change in immunostaining pattern from a mitochondrial spotted pattern to a cytoplasmic diffuse pattern. The both proteins starts to exhibit "diffuse" staining pattern 4 hours after UV irradiation (Fig. 12). To confirm these changes obtained in immunostaining by a biochemical method, cytoplasms were extracted from cells with or without UV irradiation. Fig. 13 shows the translocation of HtrA2 and cytochrome c (Cyt c) into cytoplasmic fraction after UV irradiation. HeLa cells were irradiated with UV (100 mJ/cm^2), and then each protein in cytoplasmic fraction was analyzed by immunoblotting using antibodies shown in the figure after indicated times in the figure. As shown in Fig. 13, in non-irradiated cells, HtrA2 and cytochrome c were not detected in a cytoplasmic extract. However, 4 hours after UV irradiation, HtrA2 and cytochrome c were observed simultaneously in the cytoplasm. These results proved that HtrA2, similar to Smac, was a mitochondrial protein which was released into cytoplasm in response to apoptotic stimuli.

Moreover, the examination of cytoplasm extracted from the spinal cord of a mutant SOD1 transgenic mouse, which was a model of human neurodegenerative disease and

amyotrophic lateral sclerosis (ALS), revealed that HtrA2 was released into cytoplasm in association with aging, namely with progression of diseases (Fig. 14). This observation was neither obtained with the spinal cord of a wild-type SOD1 transgenic mouse nor with the pathology-free cerebellum of the same mutant SOD1 mouse. From the above, it was suggested that HtrA2 was also involved in nerve cell-death accompanying human neurodegenerative disease.

[Example 7]

Facilitation of Sensitivity of Cell to UV by HtrA2

To study the role of HtrA2 in a cell, HeLa cells were transiently transfected (gene introduction) with plasmids respectively encoding full-length HtrA2 and full-length Smac both of which were tagged with Myc at their C-terminal. Then, apoptosis was induced by UV irradiation. 0.8 μg of pcDNA3 (Mock), pcDNA3-Smac-Myc (Smac), pcDNA3-HtrA2-Myc (HtrA2) were expressed in the HeLa cell together with 0.2 μg of pEGFP plasmid, and UV irradiation (100 mJ/cm^2) was conducted 24 hours later. After the times shown in Fig. 15, cell extracts were prepared and DEVDase activity in 30 μg of extract was determined. As shown in Fig. 15, at 4 hours after the irradiation of UV 100 mJ/cm^2, caspase-3-like DEVDase activity was increased significantly in the cells transfected with HtrA2 and Smac compared to that of control cells (Du et al., (2000) Cell 102, 33-42) (Figs. 12 and 13). In Fig. 15, error bars indicated standard errors of measurements conducted in triplicate. Fig. 12 shows the results of immunostaining of HtrA2 and cytochrome c (Cyt c). By immunostaining, detection of the intracellular localization of HtrA2 and Cyt c was conducted both in an ordinary condition and in a condition at four hours after UV (100 mJ/cm^2) irradiation, using an anti-HtrA2 antibody and an anti-Cyt c antibody. These results support the speculation that HtrA2 has an apoptosis-inducing effect like Smac, and that both Smac and HtrA2 provide such an effect in a similar mechanism.

[Example 8]

Cell-Death Induction by HtrA2 Being Expressed Outside Mitochondria

IAP-independent effects of HtrA2 were studied. Mammalian expression vectors which were the same as E. coli expression vectors used in preparation of E. coli recombinant proteins, recHtrA2 (AVPS) and recHtrA2 (MVPS), but had different a tag were constructed (Fig. 6). It can be understood that HtrA2 (MVPS) did not bind to XIAP at all, but contrary to our expectation, HtrA2 (AVPS) was also hardly bound to XIAP. It is suggested that this must be caused by the posttranslational modification such as acetylation of N-terminus of HtrA2 (AVPS). Therefore, HtrA2 (AVPS) was also used in analyses thereafter. Mature HtrA2 obtained from this construct was localized in cytoplasm (data not shown).

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1 μg of pcDNA3 (Mock) or pcDNA3-mature HtrA2-Myc (mature HtrA2) plasmid was expressed in HEK293 cell together with 0.2 μg of pEGFP plasmid, then 30 hours later, GFP-positive cells were visualized by confocal laser scanning microscope (Fig. 15). To our surprise, overexpression of HtrA2 (AVPS) made the cells to exhibit apoptosis like morphological changes with a rounded or condensed shape. Thus, it was revealed that mature HtrA2 induces untypical cell-death. Further, 1 μg of pcDNA3 (Mock), pcDNA3-mature HtrA2-Myc (mature HtrA2), pcDNA3-mature HtrA2 (S/A)-Myc [mature HtrA2 (S/A), a variant in which the serine residue in its active site was substituted for alanine residue], pcDNA3-HtrA2-Myc (FL-HtrA2) or pcDNA3-Smac-Myc (FL-Smac) plasmid was expressed in HEK293 cell together with 0.2 μg of pEGFP plasmid. Then 24 hours later, the number of GFP-positive cells which had a round shape or a condensed shape was counted (Fig. 17). It was found that protease activity of mature HtrA2 was required for untypical cell-death induction. In Fig. 17, error bars indicated standard errors of measurements conducted in triplicate. Whereas, such a significant cell morphological change was not accompanied by membrane blebbing and apoptotic body formation, and further a marked nuclear morphological change was not observed as long as the observation was made with DAPI (data not shown). Moreover, most of HtrA2-expressing cells were not dead as long as the observation was made with a Trypan blue dye exclusion tests. Even at 60 hours after the transfection, Trypan blue positive cells both in Mock and HtrA2-expressing cells were 5% or less of all transfected cells. HtrA2-overexpressing cells thus excluded the dye; however it was observed that once changes occured, they did not proliferate or did not recover, and finally detached from the plates and floated within 100 hours after transfection. It was suggested that this untypical "cell morphological change" must be an untypical "cell-death." The plasmids given in Fig. 18 were expressed in HEK293 cell together with pEGFP plasmid by the method in the same manner indicated in the examination which results were shown in Fig. 17, and 24 hours later, the cell extract was prepared and DEVDase activity in 100 μg of extract was determined. In Fig. 18, error bars exhibited standard errors of measurements conducted in triplicate. The plasmids shown in Fig. 19 were expressed in HEK293 cell together with pEGFP plasmid by the method in the same manner indicated in the examination which results were shown in Fig. 17. During the transfection and the incubation thereafter, as shown in Fig. 19, 100 μM of zVAD-fmk which was a broad range caspase inhibitor was added. In Fig. 19, error bars indicated standard errors of measurements conducted in triplicate. In the cell extract of a Bax overexpressing cell, a positive control, the caspase activity was detected more than three times as much as that of the cell extract of negative control. In contrast, in the cell extract of an HtrA2 overexpressing cell, the caspase activity only at a background level was

detected. Further, the cell-death induced by HtrA2 was not able to inhibit by zVAD-fmk and XIAP. At that time, regarding to the positive control Fas, cell-death was markedly inhibited by both inhibitory factors (Fig. 19). It should be noted that inactive type variants wherein the active site of serine protease was mutated with alanine from serine lost most of the cell-death inducing effects (Fig. 17). From these data, it was revealed that the caspase inhibitor did not inhibit the Htr2 effect, and that mature HtrA2 overexpressed outside mitochondria was capable of inducing caspase-independent and serine protease activity-dependent cell-death.

A unique feature that differs HtrA2 from Smac is that overexpression of mature HtrA2 in cytoplasm induces untypical cell-death. A cell which overexpresses HtrA2 in cytoplasm exhibits marked cell rounding and shrinkage, but does not show membrane blebbing and apoptotic body formation, further cell membrane of which is not broken. This HtrA2 induced cell-death is not inhibited by caspase inhibitors; however, the cell-death inducing effect is lost in HtrA2 variants that do not have the serine protease activity (Figs. 17 and 18).

In present Examples, as mature HtrA2 having an N-terminus which would not be modified in cytoplasm was not expressed, it is unclear whether cell-death induced by HtrA2 is suppressed by binding thereof to XIAP. However, the fact that recombinant XIAP protein was not able to inhibit the serine protease activity of mature HtrA2, suggests that XIAP inhibits cell-death induced by HtrA2 regardless of the strength of binding capacity to HtrA2.

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Industrial Applicability

As shown in Examples, HtrA2 protein of the present invention is a cell-death inducing factor participating in an unknown type of cell-death to date, and is useful in screening for a remedy for a disorder caused by excessive cell-death and in screening for a remedy for a disorder caused by aberrant suppression of cell-death.

All the publications cited herein are incorporated herein by reference in their entireties. Further, it would be appreciated by persons skilled in the art that various modifications and changes may be made to the present invention without departing from the technical concept and the scope of invention described in claims appended. Embody of those modifications and changes is intended in the present invention.